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ORIGINAL RESEARCH ARTICLE

Detection and characterization of *Kodamaea ohmeri* associated with small hive beetle *Aethina tumida* infesting honey bee hives.

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Summary

Honey bee colony infestation by the small hive beetle (SHB) is associated with fermentation of hive materials. Pollen, beetles, and robbing bees (ten of each) were collected from hives infested with SHB in both Florida and Kenya. Plating of homogenized bodies of beetles and bees and comb swabs resulted in smooth cream-colored yeast colonies that formed pseudomycelial cells as they aged. Fatty acid profiles of yeast isolates from Florida and Kenya most closely matched the profiles of *Candida krusei* and *C. sake*, respectively. The DNA sequence of the 28S and 5.8S-ITS2 of both the Florida and Kenya isolates were, however, 99-100 % homologous to *Kodamaea ohmeri*. The ITS1 region differed between the two geographic strains. The two strains produced similar volatile profiles which were attractive to SHB and contained compounds also found in honey bee alarm pheromone.

Detección y caracterización de *Kodamaea ohmeri* asociada a la infestación de colmenas de abejas por el pequeño escarabajo de las colmenas *Aethina tumida*.

Resumen

La infestación de las colmenas de abejas por el pequeño escarabajo de las colmenas (PEC) está asociada a la fermentación de los materiales de las colmenas. Se recolectó polen, escarabajos y abejas pilladoras de colmenas infestadas con PEC en Florida y Kenia. El cultivo en placas de homogeneizados del cuerpo de los escarabajos y de las abejas, y de frotis de los cuadros resultó en colonias de levaduras de color crema que formaron células pseudomiceliales cuando crecieron. El perfil de ácidos grasos de los aislados de levaduras de Florida y Kenia se aproximó mucho al de *Candida krusei* y *C. sake*, respectivamente. Sin embargo, las secuencias de ADN de las regiones 28S y 5,8S-ITS2 de los aislados de Florida y Kenia fueron 99-100% homólogas a *Kodamaea ohmeri*. La región ITS1 fue diferente según su origen geográfico. Las dos variedades produjeron perfiles volátiles similares que son atractivos para el PEC y contenían compuestos que también se encuentran en la feromona de alarma de las abejas.

Keywords: *Aethina tumida*, Small Hive Beetle, *Apis mellifera*, *Kodamaea ohmeri*, yeast, volatile attraction

Introduction

Yeasts occupy a wide variety of habitats where sugars are available, such as flowers, insect honeydew, and certain plant material. Insects present in these habitats may acquire (and vector) the various yeasts. Yeasts have been found in association with a broad range of insects, including lacewings, bees, and beetles. In many cases, consistent yeast / insect associations are observed over a large geographical region. The insect appears to benefit nutritionally from these yeasts, and it has been suggested by Suh et al. (2005) that their presence allows insects to survive on nutrient limited substrates. Yeasts have commonly been found in the digestive tract of sap beetles (family: Nitidulidae) that feed on flowers, decaying fruit, fermenting plant juices, and mushrooms (Lachance and Bowles, 2002; Suh et al., 2004; Suh, et *al.*, 2006). The yeasts found associated with flower-feeding nitidulids, including species of *Metschnikowia*, *Wickerhamiella*, and *Kodamaea* (Lachance and Bowles, 2002; Lachance et *al.*, 1999; Lachance et *al.*, 1998), are distinct from those yeasts (various *Candida* species) associated with mushroom feeding sap beetles such as *Pallodes* spp. (Suh et *al.*, 2006). Significantly, yeast associated fermentation volatiles attract certain nitidulids (Nout and Bartelt, 1998; Williams et *al.*, 1992) and serve as oviposition cues for these beetles (Phelan and Lin, 1991).

Recently, the small hive beetle (SHB) *Aethina tumida* (Coleoptera: Nitidulidae) a nest parasite of honey bees native to Africa, has invaded the United States and Australia (Neumann and Elzen, 2004). The European honey bee used for pollination in the USA is particularly susceptible to SHB. The beetles feed on the pollen, brood, and honey, and infestations destroy the colony. Fermentation of honey in the comb is associated with mass SHB infestation. From initial sampling of infested hives and SHB, we isolated a yeast species and identified it as *Kodamaea ohmeri*. The yeast grows prolifically on honey bee combs infested with SHB and this growth produces volatiles attractive to SHB (Torto et *al.*, 2007). Interestingly, the volatiles overlap with those identified as alarm pheromone components of honey bees. It is unknown whether SHB or the honey bee is responsible for the introduction of these yeasts into the hive. Because yeasts are commonly found in flowers, bees, and nitidulids, and because either can become contaminated during the common behavioural interactions between honey bees and SHB, either or both routes of entry are feasible.

Reliable procedures to detect and identify *K. ohmeri* in various biological samples are necessary to further define the interactions of this yeast with SHB and honey bees. Herein, we describe how K. ohmeri was detected in samples from hives of both European and African honey bees and associated SHB and identified using a combination of morphological, molecular, and chemical characters.

Materials and Methods

Isolation and cultivation of the yeast in infested and healthy hives

Yeast samples were collected from SHB from four European honey bee hives in Florida and from four African honey bee hives maintained at the International Centre of Insect Physiology and Ecology (ICIPE) (Kenya). SHB larvae and adults were removed from an infested hive and surface-sterilized by immersion in 70% ethanol for ~30 seconds followed by two rinses in sterile water. Homogenates of washed beetles were prepared in sterile water and resulting suspensions streaked for isolation on Sabouraud Dextrose Agar plus 1% yeast extract (SDAY). In addition to insect homogenates, a series of honey and pollen samples were collected from the infested hive and inoculated on plates. Sampling tools (metal spatula, forceps, micropestle) were sterilized with alcohol between individual samples. Inoculated plates were incubated at 28°C for 1–3 days.

Individual colonies were selected and sub-cultured on SDAY and incubated at 28°C. Selected isolates were inoculated into Durham tubes containing autoclaved bee pollen broth (1% aqueous pollen) and incubated at 3°C for five days.The gas-producing L-27 isolate produced a colony morphology characteristic of the majority of yeast colonies observed in the initial isolation.To typify growth on different media types, the L-27 isolate was plated on pollen agar (1% pollen plus 1.5% agar), Lee's agar, Czapek-Dox broth, malt extract agar, M40Y agar (a high sucrose, osmotic-stress medium), and moistened autoclaved pollen. Inoculated plates were incubated at 28°C for 7–10 days

Fatty acid profile typing

Selected isolates representative of the major colony phenotype detected from both larval and adult SHB homogenates were sub-cultured on SDAY (two isolates each from Florida and Kenya hives). Cells were harvested (80 mg wet weight) and treated chemically to extract and convert the fatty acids present in the cell wall or cell membrane fractions to fatty acid methyl esters (FAMEs) (Anonymous, 1993). Total cellular fatty acid methyl esters were subjected to gas chromatographic (GC) analysis. Resulting profiles were analyzed with the Microbial Identification System (MIDI Inc.) that matched the test organism's profiles to the available yeast FAME databases using Sherlock Version 4.5 software. Similarity indices were calculated: indices of 0 to 0.3, 0.3 to 0.6, and 0.6 to 1.0 were considered as poor, possible, and excellent matches, respectively.

DNA extraction and PCR amplification

Eight isolates from the Florida hives and two isolates from the Kenya hives were inoculated in SMY broth and incubated overnight at 26°C. Cells were pelleted and DNA was extracted using the Masterpuretm Yeast DNA purification kit (Epicentre, Madison, WI). The quantity and quality of the DNA preparations were evaluated using ethidium bromide stained agarose gels. Aliquots of DNA were amplified with a mixture of Taq DNA polymerase (Promega) and PFU polymerase (Stratagene), using the primers TW81 and AB28 for the ITS-5.8S (Curran, et *al.*, 1994) and NL-1 and NL-4 primers for the 5? divergent domain (the D1/D2 region) of the LSU rDNA (Kurtzman and Robnett,

1997). PCR products were extracted using QIAquick PCR extraction kit (Qiagen) and each PCR-amplicon bidirectionally sequenced using an ABI Prism DNA Sequencer at the Interdisciplinary Center for Biotechnology Research Core Facility at the University of Florida, Gainesville, FL. DNA sequences were compared to those deposited in GenBank with BLAST (blastn) using the default settings.

Volatile production, collection, and analysis

To compare the yeast strains collected in Florida and Kenya, bee-collected pollen (20 g) was inoculated with the L-27 clone from the Florida hive and the A-1 clone from the Kenya hive. To compare the effect of substrate, three different sterilized media (20 g each) were inoculated with the L-27 strain (collected from SDAY plates, diluted in sterile water): 1) moistened pollen, 2) moistened commercial pollen substitute Bee-Pro® (Mann Lake; Hackensack, MN, USA) and 3) SDAY (Sabouraud Dextrose Agar plus 1% yeast extract).

Inoculated and non-inoculated plates were sealed with Parafilm® and incubated at 28°C for 7 days. After incubation, the Parafilm® seals and lids were removed and the plates were placed in a glass aeration chamber (46-cm-long x 19-cm-wide) (Torto *et al.*, 2007). Volatiles were collected by passing charcoal-filtered and humidified air at 0.5 l/minute over the plates in an aeration chamber and then through Super-Q adsorbent traps (30 mg, Alltech; Nicholasville, KY, USA), which trapped the volatiles, for 1 hour at room temperature. Chemicals were eluted from traps with 150 µl methylene chloride and 174 ng of butyl butyrate were added as an internal standard to 40 µl of the extract. Samples (1 µl) were analyzed by gas chromatography on a HP-6890 equipped with a HP-1 column (30 m x 0.25 mm ID x 0.25 µm; J &W Scientific; Folsom, California, USA) linked to a HP 5973 mass spectrometer using electron impact mode (70 eV, Agilent; Palo Alto, CA, USA), with helium as the carrier gas. Samples were injected in splitless mode at an injector temperature of 240°C, and a split valve delay of 0.5 minutes. The oven temperature was held at 35°C for 1 minute, then programmed at 10°C / minute to 230°C and held at this temperature for 10 minutes. The ion source temperature was 230°C. Volatile compounds were identified by comparison of their chromatographic retention times and mass spectra to those of commercially available standards analyzed on the same instrument.

Results

Yeast prevalence and morphology

Preparations of larval and adult SHB, comb material (pollen, honey), and associated robber bees (from heavily infested hives) produced a lawn of uniform yeast colonies within 36 h when plated on SDAY. Limited spot dilution plating of these preparations demonstrated that the number of yeast colonies varied among the different samples. All of the SHB larvae (N=10) sampled from heavily infested hives consistently harboured yeasts (10^3-10^4 colony forming units or CFU / larvae) whereas 8 of 10 adult SHB harboured fewer yeasts (10-100 CFU / adult). The presence of yeast in pollen samples was variable and ranged from 0 to 100 CFU / 10 mg pollen. Interestingly, the robber bees (N=10) collected from SHB damaged hives contained yeasts at levels that ranged from 150 to more than 10^4 CFU / bee.

Isolates derived from isolation plates grew on various mycological media as well as the defined Lee's media, pollen agar, and moistened pollen. Scant growth was observed when these cultures were plated on either Czapeks-Dox or M40Y agar plates. Young 1–4 day old SDAY cultures appeared as uniform cream-colored, smooth colonies (Fig.1A). These colonies contained globose to ellipsoidal multi-polar budding yeast cells, ranging from 2-7 µm in diameter with the majority of cells under 4 µm in diameter (Fig. 2B). As the cultures aged, pseudomycelial cells formed changing the colony morphology (Fig. 1B). Within five days post-inoculation, crenellated colonies with an undulate surface formed on SDAY plates. The pseudomycelium consisted of chains of cylindrical cells 2–3 µm in diameter(occasionally swollen larger) by 9–20 µm in length (Fig. 2C)Small raised scars

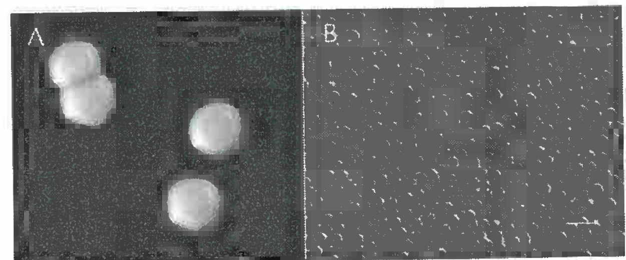

Fig 1. Smooth colony phenotype (A) of the L-27 isolate incubated for 3 days at 26°C on SDAY. Differential interference contrast (DIC) micrograph of cells (B) harvested from smooth colony. Note presence of numerous ovoid to oblong shaped yeast cells. Bar represents 10 microns.

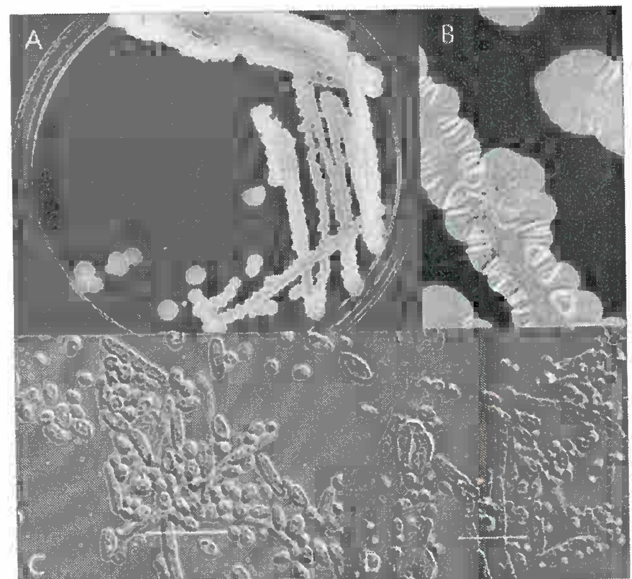

Fig 2. The L-27 cultures after 7 days on SDAY at 26°C produce a matte-like, wrinkled colony phenotype (A, B). DIC images of the cells (C, D) reveal the presence of elongate hyphal bodies intermixed with round oblong cells. In certain field, the base of these cells appeared to be swollen (D). Bars represent 15 microns.

Table 1. Summary of FAME profiles of the Florida (L-28) and Kenya (A-1) yeast isolates. Note the difference in library match between these geographical isolates. RT = retention time.

Yeast L-28

RT	Peak Name	Percent
1.652	SOLVENT PEAK	-------
1.753		-------
7.185	14:0	0.65
8.715	15:0	0.21
9.995	16:1 Cis 7 (_9)	0.39
10.067	16:1 Cis 7 (_7)	5.32
10.37	16:0	13
11.733	17:1 Cis 9 (_8)	0.98
13.364	18:2 CIS 9,12/18:0_	20.02
13.457	Summed Feature 8*	57.82
13.846	18:0	0.98
14.228	Summed Feature 10*	0.35
16.055	18:0 2OH	0.27

Yeast A-1

RT	Peak Name	Percent
2.323	SOLVENT PEAK	-------
2.583		-------
2.646		-------
7.705	14:0	1.49
10.546	16:1 Cis9(_7)	10.09
10.846	16:0	16.3
12.2	17:1 Cis9(_8)	0.91
13.815	18:2 CIS 9, 12/18:0_	25.29
13.906	Summed Feature8*	43.15
14.301	18:0	1.65
14.674	Summed Feature10*	1.12

Library Results (L-28)

YST28 3.80	0704, *Candida krusei* krusei krusei*
YSTCLN 3.80	0.828, *Candida krusei*

Library Results (A-1)

YST28 3.80	0763, *Candida sake*

*Summed features consist of the following fatty acids, which could not be separated. Summed Feature 8: 18:1 CIS9 (_9) and/or 18:1 (_8); Summed Feature 10: 18:1 Cis 9 DMA and/or an unknown (ECL 18.218).

on pseudomycelial cells indicated the prior formation of yeast cells and / or the prior points of attachment for branches. Adjacent cells were separated by constrictions, not by formal septal structures. The *K. ohmeri* strains isolated from the hives reproduced asexually by multilateral budding. Attempts at crossing different isolates on MEA agar plates did not result in ascospore formation.

Fatty acid methyl ester (FAME) analysis

MIDI analysis associated both the Florida (L-27, L-28) and Kenya (A-1, A-2) isolates with species in the genus *Candida* (Table 1). Fatty acid profiles generated from cultures of the L-27 and L-28 strains were most similar to the profiles of the Candida krusei reference (similarity indices 0.704 and 0.828). The profiles of Kenya isolates A-1 and A-2 were most similar to the profiles of *C. sake* and *C. valida* (similarity index 0.763 and 0.621).

DNA analysis

PCR-amplification of the 28S (D1/D2) and ITS1-5.8S-ITS2 of the Florida L-27 isolate produced 509 bp and 377 bp fragments (Fig. 3). The 28S and ITS1-5.8S-ITS2 DNA sequences for the L-27 isolate have been submitted to the GenBank database and assigned accession numbers AY911384 and AY911385 respectively. The ITS1-5.8S-ITS2 DNA sequence for the A-1 isolate has also been submitted to GenBank (accession number

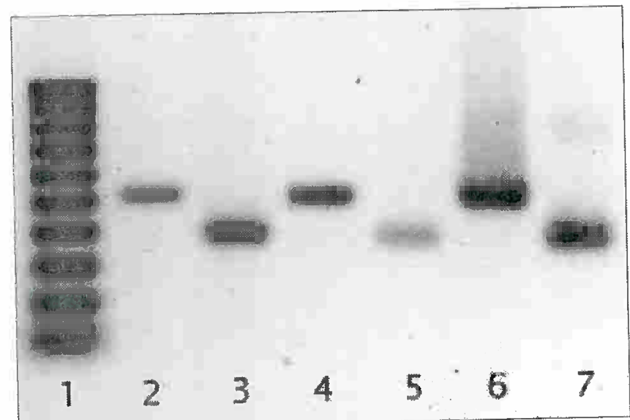

Fig 3. Ethidium bromide stained gel of the Florida L-27 (lanes 2, 3), and the Kenya A-1 (lanes 4, 5) and A-2 (lanes 6, 7) amplified with primers for the D1/D2 region of the 28S rDNA (lanes 2, 4, 6) and the ITS1-5.8S-ITS2 rDNA (lanes 3, 5, 7). Lane 1 contains molecular weight markers (100-1000 bp).

EU569326). BLAST searches (Altschul *et al.*, 1997) of both the partial 28S and ITS1-5.8S-ITS2 sequences produced matches with extremely low Expect (E) values. The 509 bp D1/D2 sequence was identical to the culture established from the type strain of Endomycopsis ohmeri (= *Kodamaea ohmeri*) (GenBank U45702),

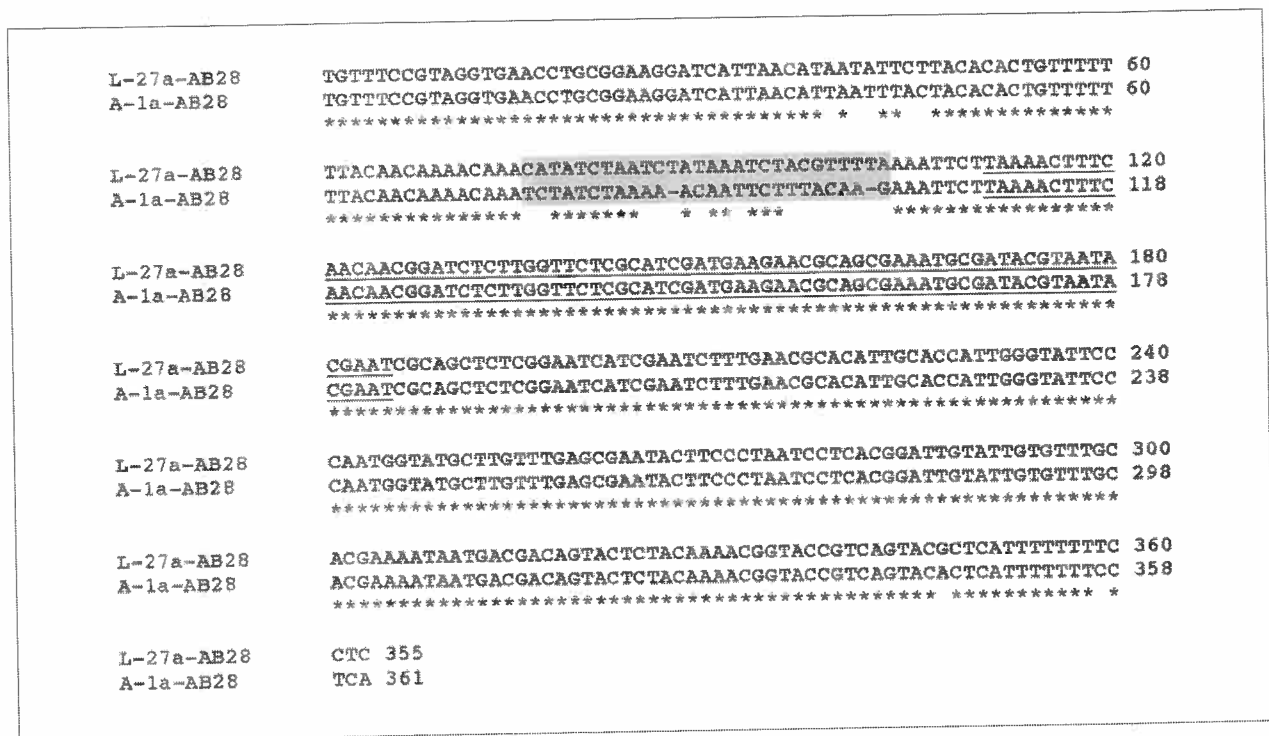

Fig 4. Alignment of the ITS1-5.8S-ITS2 sequence of the Florida (L-27) and Kenya (A-1) yeast isolates. The shaded region in the ITS2 region demonstrates the heterogeneity between the geographical isolates. The underlined sequence represents the small subunit 5.8S rDNA.

as well as other strains of *K. ohmeri* (AF335976, AY267821, AY267824), and to an unidentified yeast (AF335975). Since U45702 is the type sequence, these results suggest that the yeast we found associated with SHB honey bee hive infestation is *K. ohmeri*. Other similar sequences may be from misidentified cultures. For example, although the two *C. membranifaciens* sequences AJ508563 and AB304737 were identical to the 28S DNA sequence of L-27, the identities of these strains are suspect, as they do not match the *C. membranifaciens* type sequence (U45792). Since the 28S sequences from the Kenya A-1 and the A-2 isolates were identical to the 28S sequence of the Florida L-27 strain, these isolates were also grouped with *K. ohmeri*. Because both the 28S and ITS1-5.8S-ITS2 DNA sequences of the different isolates were identical within country of origin, isolates of the Florida L-27 and Kenya A-1 were deposited at the Agricultural Research Service Culture Collection (Accession number NRRL Y-48473).

Further confirming the identity of L-27 as *K. ohmeri*, the 5.8S sequence was 99-100% homologous to various K. ohmeri (AY168786, AF219004, AF218977) and several unidentified yeast isolates (AF536211, AF536209). The ITS2 and 5.8S regions and the short sequences of the flanking 18S and 28S subunits of the Florida and Kenya isolates were highly similar (Fig. 4). However, the ITS1 region of the L-27 sequence was distinct from both Kenya isolates, varying by 13 base pairs and 2 deletions. Interestingly, it was the Kenya ITS1 sequences that had 100% homology to available *K. ohmeri* BLAST database sequences.

Comparing the results of the FAME and BLAST analyses, the Florida L-27 sequence of the highly conserved 5.8S gene had only 78% homology to available *C. krusei* sequences. Furthermore, the D1/D2 region of different *C. krusei* 28S genes (AY305680, AY305674) had little homology to corresponding sequences generated from the SHB yeasts. The Kenya A-1 sequence of the conserved 5.8S gene had 80% homology to available *C. sake* sequences. Furthermore, the D1/D2 region of different *C. sake* 28S genes had little homology to the corresponding A-1 sequence.

Analysis of volatiles released from yeast

Yeast samples from both the Florida and Kenya hives (L-27 and A-1) released a complex of volatiles when grown on media containing pollen. These included: 3-hydroxy-2-butanone, 2- ethyl propionate, 3-methyl-butan-1-ol, 2-methyl-butan-1-ol, ethyl-2-methyl propionate, 2-methylpropyl acetate, ethyl butyrate, ethyl-2-hydroxy propionate, isopentyl acetate, 2-methylbutan-1-yl acetate, 2-heptanone, ethyl pentanoate, 3-octanone, 2-octanone, ethyl hexanoate, ethyl 3-hexenoate, hexyl acetate, and ethyl heptanoate (Fig. 5). As shown in Fig. 5, the ratios of the compounds released by L-27 and A-1 were essentially identical. SDAY media inoculated with yeast released only 3-methyl-butan-1-ol and 2-methyl-butan-1-ol, and the amounts released were significantly lower than those released by yeast growing on media containing pollen (Table 2). Interestingly, these same volatiles were collected from yeast growing on Bee-Pro plus SDAY media and non-inoculated Bee-Pro plus SDAY media. SDAY media alone produced none of the volatiles identified as being released by yeast (Table 2).

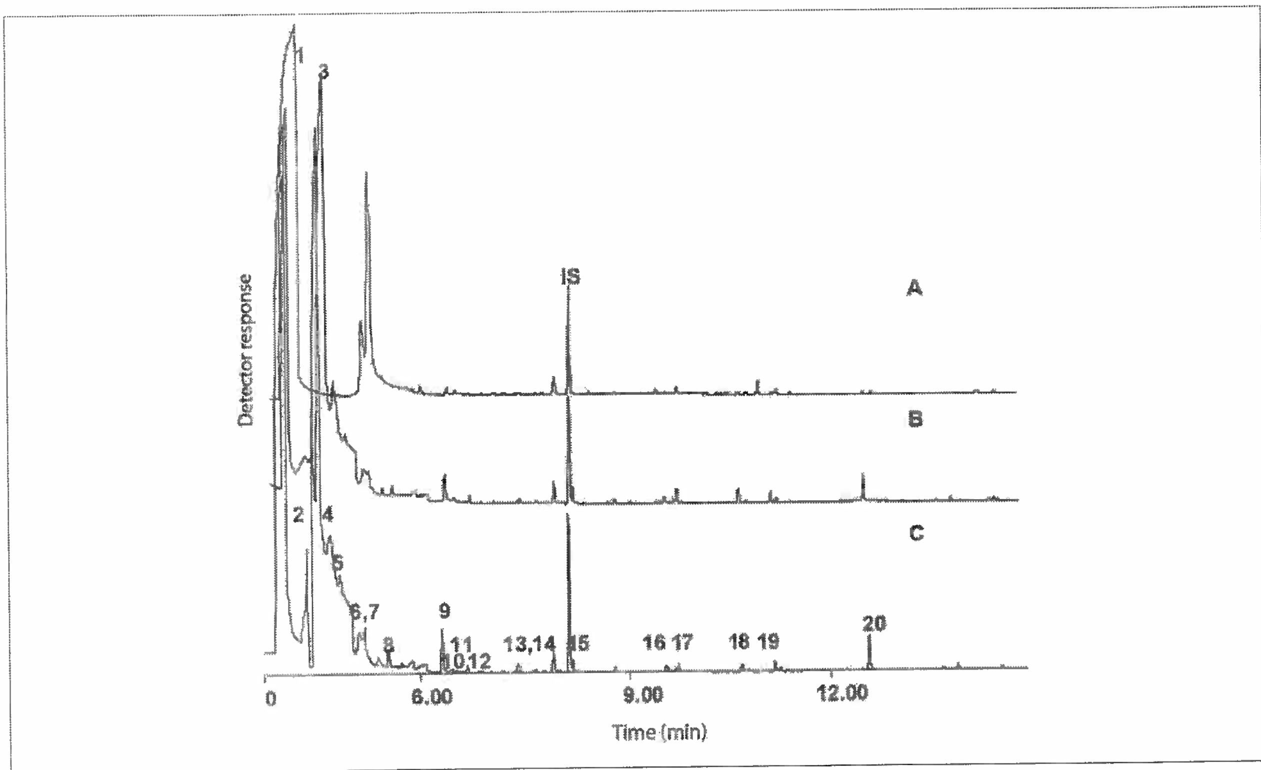

Fig 5. Representative total ion chromatograms of Super Q volatile extracts of control and yeast-inoculated media. A) Sterilized bee-collected pollen control, B) sterilized bee-collected pollen inoculated with yeast (A-1) from larvae of the SHB from African honey bee colonies in Kenya, and C) sterilized bee-collected pollen inoculated with yeast (L-27) from larvae of the SHB from European honey bee colonies in Florida. 1- 3-hydroxy-2-butanone, 2- ethyl propionate, 3- 3-methyl-butan-1-ol, 4- 2-methyl-butan-1-ol, 5- ethyl-2-methyl propionate, 6-2-methylpropyl acetate, 7- ethyl butyrate, 8- ethyl-2-hydroxy propionate, 9- isopentyl acetate, 10- 2-methylbutan-1-yl acetate, 11- 2-heptanone, 12- ethyl pentanoate, 13- 3-octanone, 14- 2-octanone, 15- ethyl hexanoate, 16- ethyl 3-hexenoate, 17- hexyl acetate, 18- ethyl heptanoate, 19- unknown, 20- unknown. IS = butyl butyrate, the internal standard.

Table 1. Volatiles collected from SDAY media either inoculated with yeast or not and containing various supplements. (+++) indicates high levels of compound, (+) indicates amounts less than 20% of +++, and (-) indicates no detectable amount.

Compound	**SDAY+ Yeast+ Pollen**	**SDAY+ Yeast**	**SDAY+ Pollen**	**SDAY+ Yeast+ Bee-Pro**	**SDAY+ Bee-Pro**	**SDAY**
3-hydroxy-2-butanone	+++	+	+	+	+	-
Ethyl propionate	+++	-	-	-	-	-
3-methyl-l-butanol	+++	++	-	+	+	-
2-methyl-l-butanol	+++	+	-	+	+	-
Ethyl-2-methyl propionate	+++	-	-	-	-	-
2-methylpropyl acetate	+++	-	-	-	-	-
Ethyl butanonate	+++	-	-	-	-	-
Ethyl-2-hydroxy propionate	+++	-	-	-	-	-
Isopentyl acetate	+++	-	-	-	-	-
2-methyl-1-butanol acetate	+++	-		-	-	-
2-heptanone	+++	-	+	+	-	-
Ethyl pentanoate	+++	-	-	-	-	-
3-octanone	+++	-	-	-	-	-
2-octanone	+++	-	-	-	-	-
Ethyl hexanoate	+++	-	-	-	-	-
Ethyl-3-henenoate	+++	-	-	-	-	-
Hexyl acetate	+++	-	-	-	-	-
Ethyl heptanoate	+++	-	-	-	-	-

Discussion

In general, the samples from honey bee hives heavily infested with SHB yielded a lawn of morphologically consistent yeast colonies. Similar samples from healthy hives yielded a mix of filamentous fungi and yeasts (Benda, unpublished). The predominant colony phenotype detected in the SHB hives (and identified as *K. ohmeri*) was similar to that of the *K. ohmeri* isolate described by Suh and Blackwell (2005). Failure to induce ascospore formation by crossing different isolates is consistent with observations by Suh and Blackwell (2005). These researchers found no ascospore formation when crossing various *K. ohmeri* strains. The sequences AJ508563 and AB304737 were identical to the 28S DNA sequence of L-27, and therefore should be assigned to *K. ohmeri* rather than *C. membranifaciens*. The problem is almost certainly the result of a nomenclatural problem, but because there is no mechanism for third-party annotation of GenBank entries, the original submitters have been notified and must be relied on to make the corrections.

Isolates sampled from the SHB infested European honey bee hives and from the SHB infested African honey bee hives produced distinct FAME profiles that were matched with different yeasts. These results highlight strain differences between the isolates from Florida and Kenya. It should be noted that minor changes in nutrient inputs has been reported to modulate fatty acid profiles (Kellogg *et al.*, 1999). This, and the absence of large numbers of insect-associated yeasts in the FAME database, may account for differences in the FAME profiles and in the differences between the FAME and BLAST search results of the strains we isolated. The marked differences at sites in the ITS1 region of the DNA sequence provide additional support of strain differences between the Florida and Kenya isolates.

Limited sampling has demonstrated an association between the yeast, SHB, and honey bee hive in two distant locations (Florida, USA and Kenya). The yeast growing on pollen sources produces bee alarm pheromones that serve to recruit additional SHB (Torto *et al.*, 2007). Interestingly, the yeast produced these attractants only when grown on media containing pollen and not on media containing the commonly used pollen substitute Bee-Pro, which lacks pollen. Pollen collected by bees may contain factors required for the yeast to produce the alarm pheromones and other volatiles attractive to beetles.

How the yeast initially enters the hive is not well understood. Potentially, adult beetles originating from heavily damaged hives or from some unknown substrate inoculates the healthy hives with yeast residues. Association of *K. ohmeri* with two other nitidulid species has been documented (Suh and Blackwell, 2005). Other yeasts may also be associated with SHB. The quantification and identity of these other yeasts and the quantification of *K. ohmeri* on SHB should be resolved. Although many insect-yeast associations have been identified, the type of interaction (mutualism, commensalism, or even competitive) is rarely understood. Understanding the relationship between SHB and *K. ohmeri* will help in developing control strategies for SHB. The attractiveness of yeast-inoculated pollen to SHB (Torto *et al.*, 2007) indicates a relationship beneficial to the beetle. Other yeasts (and other microorganisms) are found in healthy hives, but only K. ohmeri was present at the height of SHB infestation. What prevents other yeasts from growing under these circumstances is not understood and calls for investigation.

Acknowledgements

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